Biochemical Pharmacology, Vol. 35, No. 7, pp. 1199–1201, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

SHORT COMMUNICATIONS

Kinetics and substrate specificity of human and canine cytidine deaminase*

(*Received* 20 *April* 1985; *accepted* 15 *August* 1985)

1-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC†) is one of a series of 5-substituted, 2′-fluoro-2′-deoxyarabinofuranosyl cytosine analogs which inhibits Herpes virus replication *in vitro* and *in vivo* [1–3]. The selectivity of these nucleosides for virus-infected cells is due to preferential phosphorylation by virus encoded thymidine kinase [4]. The analog triphosphates are specific inhibitors of virus encoded DNA polymerase [5] and are incorporated into DNA [6]. Chromatographic analysis of DNA from virus-infected Vero cells indicates that FIAC radioactivity is incorporated as the deaminated metabolite, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) [6]. When FIAU was evaluated for antiviral effects, it was found to be as potent as FIAC [1–3]. This suggests that the pharmacologic effects of these 5-substituted cytosine analogs may be due in part to the corresponding deaminated metabolites.

Studies *in vivo* have shown marked species differences in the extent of deamination of FIAC, e.g. FIAC is poorly deaminated by dogs [7] but rapidly deaminated by humans in whom FIAU and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)uracil (FAU) are major metabolites [8]. Camiener found low cytidine (Cyd) deaminase activity in dog liver homogenates compared with human liver homogenates [9] and demonstrated a similar species difference in the deamination of 1-β-D-arabinofuranosylcytosine (Ara-C) [10]. Because of the importance of Cyd deaminase in the pharmacologic activity and toxicology of FIAC and other newly synthesized 5-substituted arabinofuranosyl cytosine analogs, we examined how these compounds served as substrates for human and canine hepatic Cyd deaminase.

Materials and methods

The 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-substituted cytosine and uracil analogs and [2-^{14}C]FIAC were synthesized by the Laboratory of Organic Chemistry at the Memorial Sloan-Kettering Cancer Center [1, 11, 12]. Cyd, deoxycytidine (dCyd) and Ara-C were obtained from the Sigma Chemical Co., St. Louis, MO. [5-^{3}H]-1-(2′-Deoxy-2′-fluoro-β-D-arabinofuranosyl)cytosine (FAC), [5-^{3}H]Cyd, [5-^{3}H]dCyd and [5-^{3}H]-Ara-C were obtained from Moravek Biochemicals, Brea, CA.

Human and canine liver samples were homogenized in 2.5 vol. of 50 mM Tris–HCl (pH 7.6). The homogenates were centrifuged at 40,000 *g* for 30 min and the pellets discarded. Dithiothreitol (DTT) was added to the supernatant fraction to a final concentration of 5 mM. The solution was heated in a water bath at 70° for 6 min and then centrifuged at 5000 *g* for 15 min. The enzyme activity was isolated by differential precipitation with saturated $(NH_4)_2SO_4$ as described previously [13]. Protein was assayed by the method of Bradford [14].

The specific activities of the enzyme preparations were determined using a procedure described previously [12, 15]. The reaction mixture contained 0.2 mM [5-^{3}H]Cyd, 6 μCi/μmole, 20 mM Tris–HCl (pH 7.6), 20 mM DTT, and the enzyme in a final volume of 500 μl. Incubation was carried out at 37° for 30 min. The reaction was started by adding enzyme and stopped by adding 2 ml of 5% perchloric acid. An aliquot of the supernatant fraction was placed on a cationic exchange column (Dowex Ag-50) and the deaminated product eluted with H_2O. The radioactivity in the supernatant fraction and in the eluant was measured with a Packard Tri-Carb model 3775 Liquid Scintillation Spectrometer using Liquiscint scintillation fluid, National Diagnostics, Somerville, NJ. The specific activity of the human enzyme was 3000 nmoles [5-^{3}H]Cyd deaminated/μg protein/30 min and of the canine enzyme was 140 nmoles [5-^{3}H]Cyd deaminated/μg protein/30 min.

The kinetic properties of human and canine cytidine deaminase were compared using radiolabeled Cyd, dCyd, Ara-C, FAC and FIAC as substrates. The reaction mixture contained 20 μM, 0.1 μCi, radiolabeled substrate, various concentrations of unlabeled substrate, 20 mM Tris–HCl (pH 7.6), 20 mM DTT and the enzyme in a final volume of 200 μl. The experimental conditions and assay for substrate and deaminated product were as described above.

The effects of substitution at the 5-position on deamination of newly synthesized 2′-deoxy-2′-fluoro-arabinofuranosyl cytosine analogs by the human enzyme were determined under the same experimental conditions, except that the substrate concentration was 150 μM. Unlabeled substrates were assayed spectrally on a Perkin Elmer model 576 Spectrophotometer using authentic substrate and the corresponding uracil analog as standards.

Results and discussion

Table 1 compares the binding affinities and relative velocities for human and canine hepatic Cyd deaminase using Cyd, dCyd, Ara-C, FAC and FIAC as substrates. The K_m values for Cyd and dCyd were similar for the human enzyme; however, both K_m values were one order of magnitude lower for the human enzyme compared with the canine enzyme. The relative V_{max} for the human enzyme with dCyd as substrate was also substantially higher than the relative V_{max} for the canine enzyme. The K_m values for Ara-C, FAC and FIAC were similar for the human and canine enzymes but were 1–2 log orders higher than the K_m values for the human enzyme with Cyd or dCyd as substrate. The relative V_{max} for the canine enzyme with dCyd, Ara-C, FAC or FIAC as substrate was also substantially lower than the corresponding relative V_{max} for the human enzyme. Thus, the lower dCyd deaminase activity for the canine enzyme compared with the human enzyme results from a higher K_m and a lower relative V_{max}, whereas the kinetics

* Supported by a fellowship grant (Dr. Fanucchi) from the JM Foundation and by funds from the National Cancer Institute (CA 08748, CA 18601, CA 18856 and CA 27569) and the Elsa U. Pardee Foundation.

† Abbreviations: FIAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine; FAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)cytosine; FFAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-fluorocytosine; FIVAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-(E)iodovinylcytosine; FBrVAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-(E)bromovinylcytosine; FMAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methylcytosine; and FEAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-ethylcytosine.

Table 1. Kinetic parameters for human and canine hepatic cytidine deaminase

Substrate	Human K_m (M)	Human V_{max} (nmoles/μg/30 min)	Canine K_m (M)	Canine V_{max} (nmoles/μg/30 min)
Cyd	$4.9 \pm 1.3 \times 10^{-5}$	5.0 ± 1.3	$3.5 \pm 1.0 \times 10^{-4}$	0.350 ± 0.120
dCyd	$5.5 \pm 0.5 \times 10^{-5}$	3.8 ± 0.3 (0.76)*	$4.5 \pm 0.2 \times 10^{-4}$	0.064 ± 0.002 (0.18)†
Ara-C	$2.7 \pm 0.8 \times 10^{-4}$	2.1 ± 0.6 (0.42)*	$5.1 \pm 0.5 \times 10^{-4}$	0.068 ± 0.005 (0.19)†
FAC	$3.3 \pm 0.1 \times 10^{-4}$	3.6 ± 0.1 (0.72)*	$6.6 \pm 0.6 \times 10^{-4}$	0.028 ± 0.001 (0.08)†
FIAC	$2.8 \pm 0.5 \times 10^{-3}$	1.4 ± 0.1 (0.28)*	$3.9 \pm 0.6 \times 10^{-3}$	0.030 ± 0.003 (0.09)†

Values are mean ± range or S.D. of two to five determinations.

* Value in parentheses is V_{max} for that compound relative to V_{max} of human enzyme with Cyd as substrate.

† Values in parentheses is V_{max} for that compound relative to V_{max} of canine enzyme with Cyd as substrate.

of Ara-C, FAC and FIAC deamination are characterized primarily by a lower relative V_{max} for the canine enzyme.

The K_m for the human hepatic enzyme with Ara-C as substrate agrees well with the values reported by Camiener [10] for the same enzyme and by Cheng *et al.* [16] for deaminase obtained from human acute myeloblastic leukemia cells. The K_m value for dCyd from this study is also similar to the value reported by Cheng *et al.* [16]; however, the K_m value for the leukemia enzyme with FIAC as substrate (3.1×10^{-4} M) is substantially lower than the K_m for the hepatic enzyme. This difference may be due to the presence of isozymes or due to differences in the techniques used to determine the kinetic constant for FIAC.

The elimination of FIAC from the plasma of patients is consistent with the results obtained from kinetic studies of human Cyd deaminase. FIAC is cleared from the plasma by deamination to FIAU and FAU, primarily in the liver [8]. Elimination follows first-order kinetics over the dosages studied [8], as would be expected for drug levels one to two log orders below the K_m for the human deaminase.

2′-Deoxy-2′-fluoro-arabinofuranosyl cytosine analogs with other substitutions at the 5-position have been synthesized in an effort to enhance selectivity for virus-infected cells [17]. The susceptibility to deamination of these compounds by the human enzyme was evaluated at 150 μM (Fig. 1) as this concentration was achievable in humans with FIAC by intravenous administration [8]. Under these conditions, substitution of fluorine, (E)halogenovinyl and methyl groups at the 5-position reduced deamination, and the 5-ethyl derivative was not deaminated.

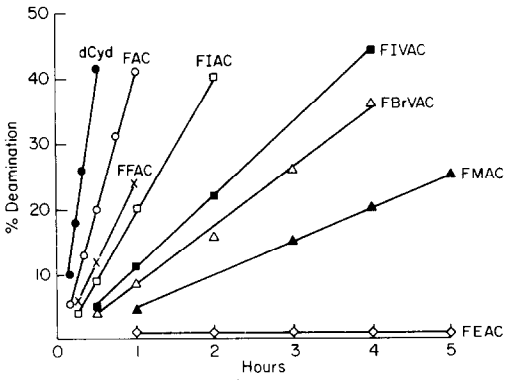

Fig. 1. Susceptibility to deamination of 5-substituted 2′-deoxy-2′-fluoro-arabinofuranosyl cytosine analogs by human hepatic cytidine deaminase. The substrate concentration was 150 μM.

In summary, it is apparent that there are marked kinetic differences in Cyd deaminase obtained from human and canine liver. This has implications for the use of dogs in toxicologic and metabolic studies of cytosine analogs. The effects of substitution at the 5-position on deamination by the human enzyme were evaluated for a number of 2′-deoxy-2′-fluoro-arabinofuranosyl cytosine analogs. Although FEAC was not deaminated, it is a potent antiviral compound *in vitro* [11, 17], suggesting that deamination may not be essential to its pharmacologic activity.

Acknowledgements—We acknowledge the technical assistance of Ms. Luan Ing Chen and the secretarial assistance of Ms. S. Sunshine.

Laboratories of Pharmacology and Organic Chemistry
Memorial Sloan-Kettering Cancer Center
Sloan-Keterring Division
Graduate School of Medical Sciences
Cornell University
New York, NY 10021, U.S.A.

MICHAEL P. FANUCCHI*
KYOICHI A. WATANABE
JACK J. FOX
TING-CHAO CHOU

* Address correspondence to: Michael P. Fanucchi, M.D., Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021.

REFERENCES

1. K. A. Watanabe, U. Reichman, K. Kirota, C. Lopez and J. J. Fox, *J. med. Chem.* **22**, 21 (1979).
2. J. J. Fox, C. Lopez and K. A. Watanabe, in *Medicinal Chemistry Advances* (Eds. F. G. Las Heras and S. Vega), Vol. 27, pp. 27–40. Pergamon Press, Oxford (1981).
3. C. Lopez, K. A. Watanabe and J. J. Fox, *Antimicrob. Agents Chemother.* **17**, 803 (1980).
4. Y-C. Cheng, G. Dutschman, J. J. Fox, K. A. Watanabe and H. Machida, *Antimicrob. Agents Chemother.* **20**, 420 (1981).
5. J. L. Ruth and Y-C. Cheng, *Molec. Pharmac.* **20**, 415 (1981).
6. T-C. Chou, C. Lopez, J. M. Colacino, A. Grant, A. Feinberg, T-L. Su, K. A. Watanabe, J. J. Fox and F. S. Philips, *Proc. Am. Ass. Cancer Res.* **24**, 305 (1983).
7. F. S. Philips, A. Feinberg, T-C. Chou, P. M. Vidal, T-L. Su, K. A. Watanabe and J. J. Fox, *Cancer Res.* **43**, 3619 (1983).
8. A. Feinberg, B. Leyland-Jones, M. P. Fanucchi, C. Hancock, J. J. Fox, K. A. Watanabe, P. M. Vidal, L. Williams, C. W. Young and F. S. Philips, *Antimicrob. Agents Chemother.* **27**, 733 (1985).

9. G. W. Camiener and C. G. Smith, *Biochem. Pharmac.* **14**, 1405 (1965).
10. G. W. Camiener, *Biochem. Pharmac.* **16**, 1981 (1967).
11. M. E. Perlman, K. A. Watanabe, R. F. Schinazi and J. J. Fox, *J. med. Chem.* **28**, 741 (1985).
12. T-C. Chou, A. Feinberg, A. J. Grant, P. Vidal, K. A. Watanabe, J. J. Fox and F. S. Philips, *Cancer Res* **41**, 3336 (1981).
13. K. A. Watanabe, U. Reichman, J. J. Fox and T-C. Chou, *Chem. Biol. Interact.* **37**, 41 (1981).
14. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
15. T-C. Chou, J. H. Burchenal, J. J. Fox, K. A. Watanabe, C-K. Chu and F. S. Philips, *Cancer Res.* **39**, 720 (1979).
16. Y-C. Cheng, R-S. Tan, J. L. Ruth and G. Dutschman, *Biochem. Pharmac.* **32**, 726 (1983).
17. J. J. Fox, K. A. Watanabe, R. F. Schinazi and C. Lopez, in *Pharmacological and Clinical Approaches to Herpes Viruses and Virus Chemotherapy* (Ed. R. Kono), Excerta Medica, Amsterdam, in press.

Biochemical Pharmacology, Vol. 35, No. 7, pp. 1201–1203, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Requirement of ADP for arachidonic acid-induced platelet aggregation: studies with selective thromboxane-synthase inhibitors

(*Received* 7 *June* 1985; *accepted* 16 *September* 1985)

Arachidonic acid induces human platelet aggregation [1]. It was originally suggested that this occurred through the formation of cyclic prostaglandin endoperoxides [2] but later on it was shown that these unstable compounds generated thromboxane A2 (TxA2), an extremely potent aggregating agent in platelets [3]. Evidence was presented that arachidonic acid-induced platelet aggregation is dependent on ADP [4–6], the major endogenous amplifier of platelet aggregation response [7]. Other studies, however, indicated direct platelet activation by cyclic endoperoxides and/or TxA2 [8–15]. From these conflicting results the role of ADP released during platelet aggregation induced by AA remains unclear. We show here that selective pharmacological blockade of platelet TxA2-synthase did not result in any apparent modification of the platelet aggregatory response to arachidonic acid, provided ADP was available. We suggest that cyclic endoperoxides, accumulating when TxA2 synthesis is selectively prevented, have an absolute requirement for ADP, while TxA2 can induce platelet aggregation independent of this nucleotide. Thus, the various amplifying pathways during platelet stimulation have more or less relevance not by themselves but rather in relation to the availability of the others.

Materials and methods

Citrated human platelet-rich plasma (PRP) was stimulated for 3 min in a Born aggregometer (Elvi 840, Elvi Logos, Milan, Italy) under constant magnetic stirring (1000 rpm) with sodium arachidonate (AA, Sigma, >99% pure) [16], or the endoperoxide stable analogue U-46619 [17] or adenosine-5′-diphosphate (ADP, Sigma) [16]. For each individual PRP sample the Threshold Aggregating Concentration (TAC) of the stimulus that induced a 70% increase in light transmission was selected [16]. PRP was preincubated for 3 min at 37° with dazoxiben (UK 37,248-01, Pfizer Central Res., Sandwich, U.K., 40 μM) or OKY-046 (Ono Pharmaceutical Co., Japan, 40 μM) or solvent (Tris–HCl, 150 mM, pH 7.4) before AA addition. Creatine phosphate (Sigma 5 mM, CP) and creatine phosphokinase (Sigma 10 U/ml, CPK) were freshly dissolved in Tris–HCl 15 mM pH 7.4. Potato apyrase prepared according to Molnar and Lorand [18] hydrolysed 2 nmoles ATP/min/μg protein. The addition of CP/CPK or apyrase was able to completely prevent 10 μM ADP-induced platelet aggregation. Ketanserin (Janssen Pharmaceutica, Beerse, Belgium) was dissolved in redistilled water.

An aliquot of PRP was deproteinized by addition of equivolume of absolute ethanol and TxB2 and PGE2 measured by specific radioimmunoassay [19]. 5-Hydroxytryptamine (5-HT) release was assayed in the platelet pellet obtained by rapid centrifugation of PRP, treated with 0.4 N HCl, sonicated and centrifuged. 5-HT was assayed in this clear supernatant by high performance liquid chromatography with electrochemical detection as described [20].

Results and discussion

Human platelet aggregation induced by AA was accompanied by generation of TxB2, the stable derivative of TxA2 and the release reaction, as measured by the release of endogenous 5-HT (Table 1). Two selective TxA2-synthase inhibitors, dazoxiben and OKY-046, completely suppressed TxB2 generation, but only partially prevented the release reaction. Cyclic endoperoxides accumulated concomitantly as suggested by the 10–20-fold increase of platelet PGE2 generation [21, 22] (Table 1).

The ADP-removing enzymatic system CP/CPK had no effect on AA-induced platelet aggregation, but almost completely prevented it in platelets preincubated with either

Table 1. TxB2 and PGE2 generation and 5-HT release in human PRP stimulated by TAC of AA (alone or in the presence of a TxA2-synthase inhibitors) or of U-46619

Release (%)	TxB2 (pmol/ml)	PGE 2 (pmol/ml)	5-HT
AA (0.4–0.6 mM)	900–2500	80–150	40–50
AA + Dazoxiben (40 μM)	6–15	700–1200	29–35
AA + OKY-046 (40 μM)	3–12	1300–1700	24–34
U-46619 (180–270 nM)	<5	<5	14–25

Figures are ranges from 6 samples.
5-HT release is % of total platelet 5-HT content [20].